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Gonadal Atresia, Estrogen-Responsive, and Apoptosis-Specific mRNA Expression in Marine Mussels from the East China Coast: A Preliminary Study

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Abstract

This preliminary survey analysed mussel atresia incidences, estrogen-responsive and apoptotic-specific molecular end points, and aqueous and gonadal levels of selected estrogens from the East China coast. Estrogen levels were low (e.g. < LOD-28.36 ng/L, < LOD-3.88 ng/g wet weight of tissue for BPA) relative to worldwide freshwater environments, but high oocyte follicle atresia incidences (up to 26.6%) occurred at selected sites. Expression of estrogen-responsive *ER2* was significantly increased in males relative to females at sites with high atresia incidences in females. A second estrogen-responsive gene, *V9*, was significantly increased at two sites in April in females relative to males; the opposite was true for the remaining two sites. Apoptosis-specific genes (*Bcl-2*, *fas*) showed elevated expression in males relative to females at the site with the highest atresia incidence. These results provide coastal estrogen levels and the utility of several estrogen-specific molecular-level markers for marine mussels.

Keywords Mytilus · Estrogen · Atresia · Apoptosis

Studies have highlighted that water, sediment and mussels from the Shanghai area of the East China coastal region contain significantly higher levels of legacy and emerging contaminants relative to other sites in China (Table S1). Cumulative evidence suggests that the Changjiang Estuary, and the east coastline of China, receives many contaminant classes similarly to coastlines worldwide (Atkinson et al. 2003; Koyama et al. 2013; Emnet et al. 2015), and the biological implications are unknown.

Mussels, Mytilus sp. concentrate contaminants in their tissues and are widely used in toxicology studies and as bioindicator species (Beyer et al. 2017). Molluscs contain vertebrate-like steroids, such as androgens (testosterone), estrogens (estrone E1 and estradiol E2), and progestins (Reis-Henriques et al. 1990; Zhu et al. 2003) and have enzymes typically involved in the steroidogenesis pathways (Janer and Porte, 2007). The presence of sex steroids is therefore established in molluscs, but their biological role is undecided (Scott, 2013). A 10-day short term exposure to synthetic estrogen 17-α ethinyl estradiol (EE2) and E2, resulted in a significant increase in estrogen receptor (*ER2*) mRNA expression in the gonad of *M. edulis* during early stages of gametogenesis (Ciocan et al. 2010). Gonad egg yolk protein VTG mRNA expression was also significantly increased in these estrogen-exposed mussels (Ciocan et al. 2010). In parallel studies, *serotonin receptor, cyclooxygenase* and *vitelline envelope zona pellucida domain 9* (*V9*) mRNA expressions have also been impacted by E2 exposure in mussels (Cubero-Leon et al. 2010; Ciocan et al. 2011). Cumulatively, these studies suggest that mussels are susceptible to exogenous sources of estrogens. In terms of the subsequent cellular and tissue level pathological impacts of endocrine disrupting chemicals (EDCs) in bivalve populations in general, there have been reports of intersex (Ortiz-Zarragoitia and Cajaraville 2010), gonadal neoplasms

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(Barber 2004), atresia (Ortiz-Zarragoitia et al. 2011) and apoptosis (Matozzo and Marin 2005). The latter results from programmed cell death and is part of molluscan immune defence; the extrinsic apoptosis pathway involves death receptors (fas, Trail, TNF) and G-protein coupled receptors, whereas the interlinked intrinsic pathway is controlled by Bcl-2 family proteins (Kiss 2010).

The aim of this study was to perform a pilot investigation into the reproductive health of marine mussels, Mytilus sp., comprising either *M. coruscus* or *M. galloprovincialis* (Qu et al. 2019; Ding et al. 2020) from the East China coastal region, at four sampling locations and for two seasons at one site (to indicate any seasonal variation). Biomarkers were adopted for biomonitoring in marine molluscs including; targeted molecular endpoints responsive to estrogenic compounds (*V9* and *ER2* mRNA expression) (Ciocan et al. 2010) and specific to apoptosis (*Bcl-2* and *fas*) (Lee et al. 1997; Morita et al. 1999). These were used alongside histology to identify atresia as a relevant biological endpoint of impact relating to reproductive health (Smolarz et al. 2017) as well as aqueous and gonadal tissue estrogen concentrations to determine exposure levels and uptake.

Materials and Methods

Mytilus sp. (n = 26–60, depending on availability) were collected at low tide from four Chinese coastline locations: Qingdao (comprising 4 local subsampling sites QD-A, QD-B, QD-C and QD-D), Yantai (YT), Shengsi (SS) and Xiamen (XM), to investigate spatial variation, and during two seasons at one sampling location (Qingdao) to investigate any temporal variation. During April 2014, samples were collected at QD-A to QD-D. During July 2014, samples were collected from YT, QD-B, QD-D, SS and XM (Fig. S1, Table S2). Mussels (n = 26–60) were measured and gonad tissues were immediately dissected into 0.5 cm^2 pieces: one was fixed in 4% formaldehyde and stored at room temperature for histology (for which n = 1 slides for each individual was analysed), one was kept in RNAlater™ (Sigma-Aldrich, USA) at − 20°C for molecular analyses, and one was stored at −80°C until chemical analyses. 3 L of seawater was taken from each site and stored at − 20°C until chemical analyses. Samples (Table S2) were also analysed blind (no knowledge of sex or development stage) for gene expressions using mussels from each site (and at QD from two seasons). Total RNAs were extracted from ~ 20 mg of each mussel gonad with RNeasy reagents (Qiagen, Germany) with a DNase I digest. rRNA integrity was determined by 1% agarose-formaldehyde gel electrophoresis. First strand cDNAs were generated from 1 µg total RNA using PrimeScript™ RT reagents (TaKaRa, Dalian, China). Real-time PCR reactions (final volume 20 µL) contained 10 µL SYBR® Premix Ex Taq, 2 µL cDNA, 0.4 µM primers, and 0.4 µL ROX Reference Dye (Takara, Dalian) (Table S3). Reference genes *18S* and *EF1a*, previously validated for stability in estrogen-exposed mussel gonads (Cubero-Leon et al. 2012), were selected for relative quantification. A control without cDNA was used to determine the specificity of target cDNA amplification. Cycling parameters were: 95°C for 30 s, 40 cycles of 5 s at 95°C and 34 s at 60°C with a 7500 RT PCR system (Applied Biosystems, U.S.A.). Melting curves and gels confirmed specificity of amplified products. The efficiency of each primer pair was calculated by cDNA dilution factors. Relative expression levels of the four target genes were calculated using the comparative ΔCT method (Livak and Schmittgen 2001), outliers were defined as twice the standard deviation. Mussel gonads were wax embedded, transversely sectioned (6 µm), stained with haematoxylin–eosin, and observed with an Olympus BX53 microscope (Japan) to determine the sex (where possible), stage of maturation (Seed 1969), and occurrence of atresia.

Aqueous stocks (1000 mg/L) of estrogen standards (E1, E2, E3 and EE2) and the internal standard (E2-d2) (Dr. Ehrenstorfer™, LGC Ltd) were diluted with methanol (10 mg/L). All solvents were HPLC grade. Water samples were extracted as described by Yan et al. (2013) and Shi et al. (2014). Briefly, water samples were filtered using preashed 0.7 µm GF/F filter and spiked with 20 ng of internal standards. Water samples were pre-conditioned with ultrapure water and methanol and passed through an Oasis HLB cartridge at a standard flow rate (5–10 mL/min) for solid phase extraction (SPE). The compounds were eluted with 10 mL of methanol then concentrated to 0.5 mL. Mussel gonads (1.5 g) were injected with 20 ng internal standards and extracted with an ASE 350 accelerated solvent extractor by a mixture of methanol and acetonitrile (1:1). The targeted EDCs were analyzed by a Waters Acquity™ UHPLC–MS/MS system according to Ye et al. (2013). Targeted EDCs were measured in negative ion mode. The flow rate of the desolvation gas (N2) was 800 L/h, and temperature was set at 500°C. The flow rate of the collision gas (Ar) was 10.2 mL/h, and capillary voltage was 2.8 V. The limit of quantification (LOQ) and limit of detection (LOD) in aqueous samples were 0.30–1.97 ng/L and 0.10–0.49 ng/L respectively, and 0.33–1.55 ng/g wet weight and 0.15–0.44 ng/g respectively in tissue samples.

Statistical analyses were performed using SPSS. The Kolgomorov-Smirnov test was used to examine normality of residuals and the homogeneity of variance. Sex ratio bias was determined using Chi squared test ($p < 0.05$). For the mRNA expression data, the Scheirer-Ray-Hare (SRH) test was used for QD sites in April to examine the difference caused by sex and sampling site and determine if the two factors interact. For all sites, significance for relative gene expression between sexes at individual sampling sites or the

same sex at different sampling seasons, was tested using the Kruskal–Wallis (KW) non-parametric test (non-normal distribution). Outliers, according to the MIQE guidelines (Bustin et al. 2009), were excluded from the statistical analysis. Significance was accepted at: $*=p<0.05$, $**=p<0.01$, $***=p<0.001$.

Results and Discussion

Following histological examination, sex and stage of gametogenesis were determined, as well as the incidence of atresia in females only (Table S2, Fig. 1). A sex ratio bias in favour of males was observed at QD-D (relative to QD-A to C) in April (Table S2). Many spent mussels were detected in July, prohibiting sex ratio calculations. No previous marine mussel sex ratio data is available for this region, though female sex ratio bias has been reported in mussels impacted by spilled oil in the Bay of Biscay (Ortiz-Zarragoitia et al. 2011), and in clams, *Scrobicularia plana*, at selected sites from the English Channel (Pope et al. 2015). A male bias has previously only been observed in *S. plana* from six locations in the English Channel region (Pope et al. 2015) and following a > 36 week laboratory exposure using the bivalve *Gomphina veneriformis* to tributyltin (Park et al. 2015). Herein Mytilus sp. were observed at various stages of gametogenesis (Table S4), a process that is seasonal and spatially dependent, and appears to reflect the variability in gametogenesis stages characteristic of the species (Seed and Suchanek 1992), yet the apparent occurrence of a sex ratio bias is unusual.

Atresia is a natural part of the gametogenesis cycle in which the ovarian follicles die, allowing the resorption of gametes at the end of the hatching stage, and a resting period before a new cycle begins. Pre-spawning oocyte atresia may also occur (Beninger 2017). A typical indicator of atresia in females is vacuolisation within eggs (Fig. 1B). The incidence of oocyte atresia varied temporally and spatially; atresia was detected in females at all QD sites in April, with the highest incidence (26.6%) observed at QD-B, yet no atresia was found in females at any sites in July (Table S2). An increased incidence of atresia has previously been reported in *M. provincialis* as a natural occurrence in the winter in Galicia, Spain as a result of unfavourable conditions for spawning after the gametes ripened (Suarez et al. 2005). High incidences of atresia have also been reported in *M. edulis* from Boston Harbor/Cape Cod Bay, U.S.A. (Kimball 1996), and *M. trossulus*, Gulf of Gdansk, Poland (Smolarz et al. 2017), as well as following experimental exposure of *M. edulis* to North Sea oil and alkylphenol (Aarab et al. 2004), metals using *Crassotrea angulata* (Vaschenko et al. 2013), and estrogens using *M. trossulus* (EE2, at 50 and 500 ng/dm^3) (Smolarz et al. 2017). In vertebrates, an increased incidence of oocyte atresia has also been reported in fish (*Clarias gariepinus* and *Chalcalburnus tarichi*) experimentally exposed to various estrogens (EE2 at 50 ng/L and 100 ng/L) (Kaptaner and Unal 2011; Sridevi et al. 2015). Exposure of medaka fish (*Oryzias latipes*) to 10 ng/L EE2 failed to induce atresia though did increase the rate of apoptosis in testicular cells (Weber et al. 2004).

Gene expression was investigated as follows: broadly across four East China Sea coastal locations; more locally within one location (four subsampling sites); and finally, also at the latter location, across two seasons (spring and summer) when the natural gametogenesis cycle in molluscs is at different stages. qPCR revealed that sex influences *V9* expression levels significantly (SRH, $p=0.000$) at QD sites in April but subsampling location (QD-A to D) does not; there was no interaction between sex and site. Female mussels at QD-A and QD-B displayed elevated *V9* expression compared with males (Fig. 2A, Table S4). *V9* expression in QD-B females was significantly higher in April than July, coinciding with the early developing and mature stages of gametogenesis (Table S4, Fig. 1A). Uncharacteristically

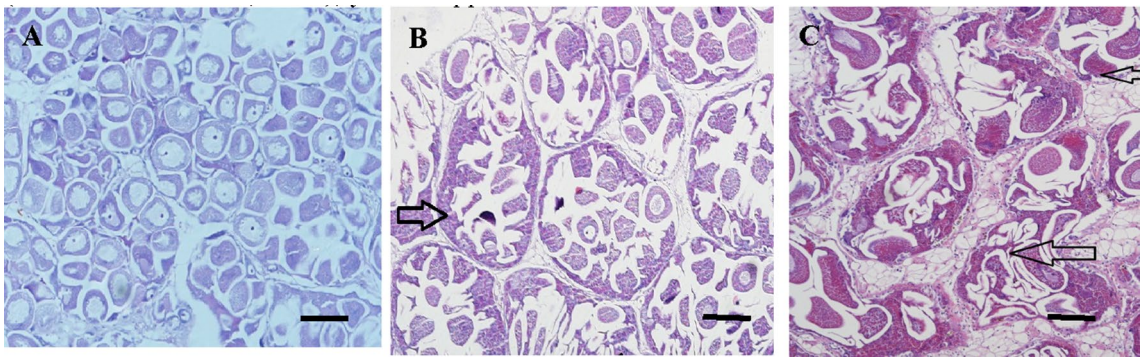

Fig. 1 Mytilus sp. gonads stained with H & E stain. **A** normal female at developing/mature stage (βIII/βIV); **B** mature female (βV/γIV) with atretic oocytes (arrows); **C** massive degeneration (atresia) of female follicles in a mature gonad. Size bar 100 µm, 200× magnification, (Seed, 1969)

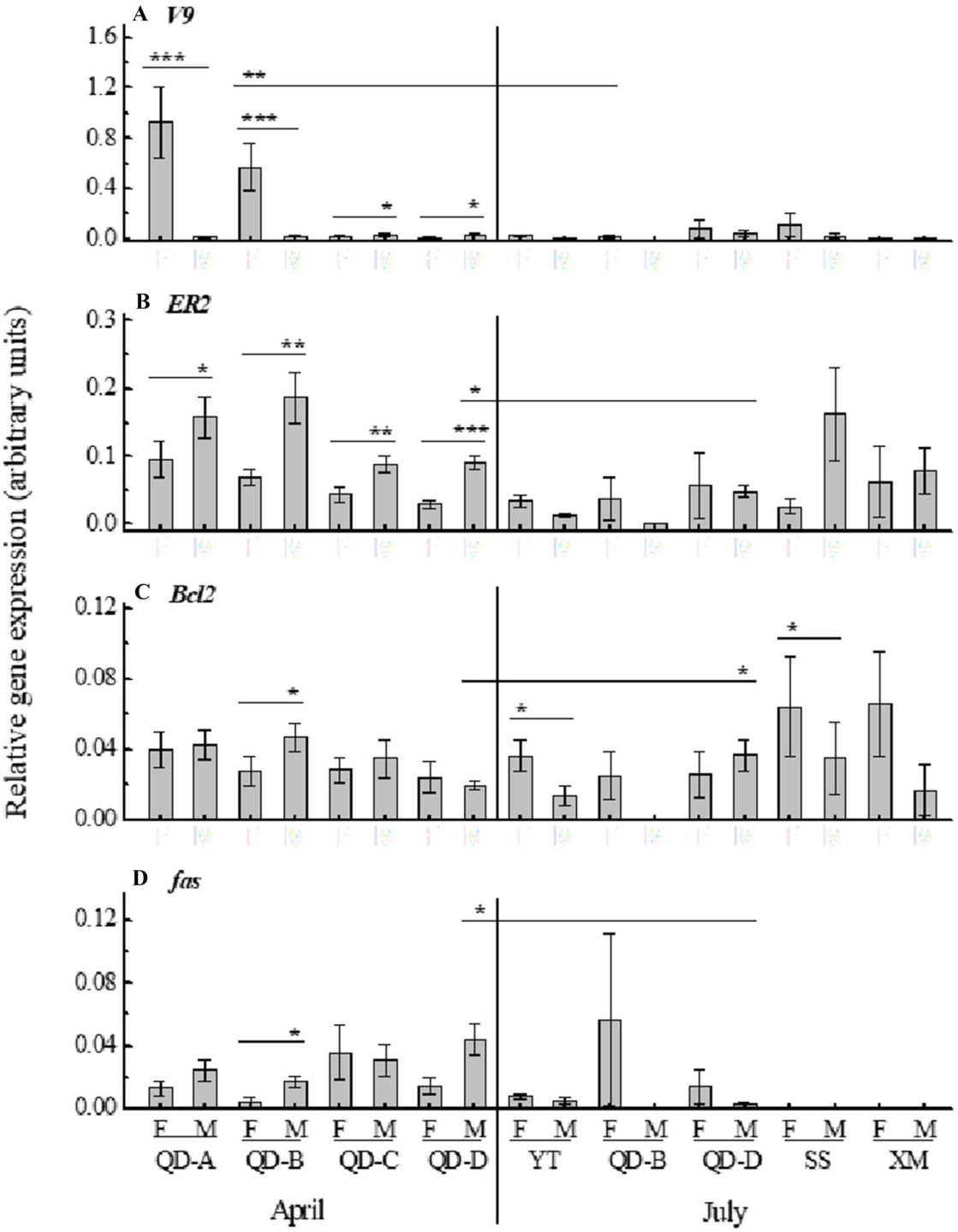

Fig. 2 Relative gene expression of target genes) *V9*, **B** *ER2*, **C** *Bcl-2*, and **D** *fas* in mussel gonad tissues taken from seven geographical sampling sites and two sampling seasons. Data are plotted as mean ± SEM (n = variable, Table S2). Lines above the bars denote significant differences (Kruskal–Wallis test): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Abbreviations of sampling sites: *QD* Qingdao, *SS* Shengsi, *XM* Xiamen, *YT* Yantai

for males, which do not normally produce eggs, *V9* expression was elevated in males compared to females at QD-C and QD-D during April (Fig. 2A), indicating induction of egg-specific cellular signalling pathways. Natural seasonal variation in egg yolk associated proteins (and associated gene expression) occurs in scallop (*Patinopectin*

yessoensis) and mussel with peaks in March/April coinciding with development and mature stages of gametogenesis and lower levels in summer coinciding with post-spawning/degeneration (Osada et al. 2003; Ciocan et al. 2010). Male mussels typically display a low background level of egg-related gene expression (Ciocan et al. 2010), yet males at QD-C and QD-D during April show significantly elevated *V9* expression compared with females (Fig. 2A). Similar increases in egg yolk (specifically *vitellogenin*, *VTG*) and membrane (*choriogenin/zona radiata*) gene expressions and protein levels in males have been reported in fish (*Oryzias melastigma)* and are utilised as biomarkers following estrogen: E2, EE2 and BPA (Chen et al. 2008), and xenoestrogen: refinery oil exposure (Arukwe et al. 1997).

For *ER2* mRNA expression, both sex (SRH, $p = 0.000$) and sampling site ($p = 0.001$) influence expression levels significantly in April. Expression was significantly higher in QD-D males in April compared to July (Fig. 2B). In addition, *ER2* is significantly increased in males compared with females at all QD sites in April (Fig. 2B), with a similar, though non-significant, trend observed at SS in July. The precise underlying mechanism resulting in the increase of *ER2* expression in male mussels, relative to females, is unclear due to the ongoing debate surrounding the functional role of ERs in bivalves (Scott 2013; Nagasawa et al. 2015). *V9, ER2* and *VTG*/VTG, have previously been recognized as up-regulated genes in *M. edulis* (Ciocan et al. 2010; Nagasawa et al. 2015), scallop *P. yessoensis* (Osada et al. 2003), and oyster *Saccostrea glomerata* (Andrew et al. 2010) exposed to estrogens (E2 and/or EE2) under laboratory conditions, and in intersex clams, *S. plana* (Ciocan et al. 2012). In contrast, *MeER1* and *MeER2* expressions were significantly downregulated in trochophore (early life) stages of the mussel *M. galloprovincialis* at 24 h post E2 (10 µg/L) and BPA (10 µg/L) exposure, although up-regulated at a lower dose of BPA (1 µg/L), suggesting dose dependent response (Balbi et al. 2016). With respect to both the *V9* and *ER2* expressions and implications of their change, it is important to consider the ongoing debate surrounding the role of estrogens in bivalve reproduction and any potential endocrine disruption. Molluscs contain vertebrate-like steroids, including E1 and E2 (Reis-Henriques et al. 1990; Zhu et al. 2003) and have steroidogenesis pathway enzymes (Janer and Porte 2007). Sex steroid presence is not doubted, but the biological role and possible disruption is debated (Scott 2013).

Linking to relevant biological endpoints of reproductive impact, *Bcl-2* (a regulator of cell death) and *fas* (encoding a protein ligand that binds to an associated receptor triggering apoptosis) expression have both been used as markers of apoptosis, including follicular atretic conditions, in vertebrates (Lee et al. 1997; Morita et al. 1999). This is the first instance of their use with *Mytilus* sp. Similar to *V9*, sex also influences *Bcl-2* mRNA expression levels significantly (SRH $p = 0.037$), but sampling site does not at QD in April; no interaction was detected between sex and site. In April, *Bcl-2* expression in females showed significant down-regulation relative to males from QD-B (Fig. 2C), which corresponded with the highest incidence of atresia (26.6%) in females at any site. *Bcl-2* expression inhibits oocyte follicle atresia in mice (Morita et al. 1999), thus a down-regulation in QD-B females may reflect the higher atretic incidence. In contrast, males collected in summer from YT and SS displayed significantly down regulated *Bcl-2* expression relative to females (Fig. 2C). In other studies, experimental E2 exposure significantly increases, while testosterone inhibits, *Bcl-2* expression (Huber et al. 1999), which corresponds with mussels from YT, then SS, having the highest tissue levels of total estrogens (Table S5). A significant increase in *Bcl-2* expression was also observed between July and April for QD-D males (Fig. 2C).

Increased *fas* expression has previously been associated with increased apoptosis in vertebrate gonadal cells (Lee et al. 1997; D'Alessio et al. 2001). Herein, *fas* expression levels were significantly influenced by sex (SRH, $p = 0.023$), but not sampling site (QD-A to D) in mussels during April, with no interaction detected between the two factors. *Fas* expression in males was significantly up-regulated relative to females sampled at QD-B during April, with a similar trend for QD-A and QD-D in April (Fig. 2D). *Fas* expression was also significantly higher in males at QD-D in April relative to July. In other species, experimental monophthalate exposure using mice has been shown to increase *fas* expression and trigger sertoli cell apoptosis (D'Alessio et al. 2001). Here, the aqueous BPA levels were relatively high at SS (28.36 ng/L, Table S5), yet no *fas* mRNA expression was observed in either sex (Fig. 2D).

The aqueous estrogen levels detected herein are relatively low (< LOD-28.36 ng/L, Table S5) compared with previous values (~ 1–200 ng/L range) from the region, and more in line with values (~ 1–30 ng/L) from other worldwide coastal locations (Table S1). Similarly, the tissue levels of total estrogens detected (Table S5) are generally lower (at ~ 0.5–3 ng/g ww) than those previously reported in Chinese coastal waters (1374–3199 ng/g lipid weight) (Zhang et al. 2011), though differing units make direct comparison problematical. The average estrogen levels (~ E2 1.94 ng/g, EE2 1.05 ng/g ww) (Table S5) in gonads are similar to Baltic Sea mussels *M. edulis-trossulus* hybrids (3.9–4.8 ng/g wet weight) (Zabrzanska et al. 2015) and Antarctic clams for E2 (0.8–2 ng/g wet weight) (Emnet et al. 2015). To compare, freshwaters receive effluents with EE2 as high as 800 ng/L (Koplin et al. 2002).

The East China coastal region is a receiving environment for many classes of contaminants (Table S1). Herein, the differential expression of reproduction-relevant, estrogen-specific biomarkers *V9* and *ER2*, indicate a potential biological

impact related to (xeno)estrogen contaminants. Future work is required to further optimise and validate these biomarker responses, as well as monitor the reproduction endpoints of mussels to determine any population level impacts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00128-022-03461-2.

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